
POLYPHYLY IN *GUETTARDA* L.
(RUBIACEAE, GUETTARDEAE)
BASED ON nrDNA ITS
SEQUENCE DATA^{1, 2}

Frédéric Achille,³ Timothy J. Motley,⁴ Porter P.
Lowry II,^{3, 5} and Joël Jérémie³

ABSTRACT

The genus *Guettarda* (Guettardeae–Rubiaceae) comprises approximately 150 species, ranging from eastern Africa through the islands of the Indian and Pacific Oceans to the Neotropics. Sequence data from the nuclear ribosomal DNA internal transcribed spacer (ITS) region were used to test the monophyly of *Guettarda* and its relationships to closely related genera within Guettardeae. The results indicate that *Guettarda* and two smaller genera, *Antirhea* and *Stenostomum*, are polyphyletic. Most *Guettarda* species fall into two distinct groups: a Neotropical lineage that also includes the widespread Indo-Pacific strand species *G. speciosa* (the type of the genus), and a New Caledonian lineage that, along with *Antirhea* and *Timonius*, comprises a dioecious Paleotropical clade. The Hawaiian endemic *Bobea*, traditionally considered close to *Timonius* and assumed to be of Old World origin, appears to be more closely related to Neotropical *Guettarda* species, suggesting that dioecy may have evolved twice within the tribe. The use of traditional gynoecium characters to delimit genera within Guettardeae is not congruent with the ITS phylogeny; other features, such as inflorescence architecture, sexual system, and palynology, appear to correlate more closely with the molecular phylogeny.

Key words: biogeography, dioecy, *Guettarda*, Guettardeae, islands, ITS, Neotropics, Paleotropics, Rubiaceae, systematics.

Rubiaceae, one of the largest families of flowering plants with ca. 13,000 species and 650 genera (Delprete, 2004), comprise 44 tribes (Robbrecht, 1988) currently assigned to three subfamilies (Bremer & Jansen, 1991; Bremer et al., 1995; Bremer, 1997; Bremer et al., 1999). The genus *Guettarda* L. and its relatives have been regarded for most of the last two centuries as a fairly clear-cut group, generally recognized as tribe Guettardeae, and sometimes even as a distinct subfamily under the invalid name Guettardoideae [= Antirheoideae] (Verdcourt, 1958; Bremekamp, 1966). While assigned to subfamily Antirheoideae by Robbrecht (1988), a series of recent molecular phylogenetic studies have shown that Guettardeae are best placed in Cinchonoideae (Bremer & Struwe, 1992; Bremer et al., 1995; Bremer,

1996; Rova, 1999; Rova et al., 2002; Andersson & Antonelli, 2005).

As part of a systematic study of the New Caledonian species of *Guettarda*, a molecular phylogenetic analysis was conducted to evaluate the monophyly of this widespread tropical genus, whose definition has varied considerably. Originally described by Linnaeus (1753) as monotypic, *Guettarda* has progressively grown with the description of additional species and the inclusion of at least five other genera: *Matthiola* L. and *Laugieria* Jacq. (Lamarek, 1792); *Antirhea* (“*Antirrhoa*,” Mueller, 1875); and, more recently, *Tournefortiopsis* Rusby and *Dicrobotryum* Willd. ex Roem. & Schult. (Standley, 1931). The broadest definition of *Guettarda* by far was that of Baillon (1879, 1880), who included all Guettardeae species

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³ Muséum National d’Histoire Naturelle, Département Systématique et Évolution, USM 602, 57, rue Cuvier, CP 39, 75231 Paris CEDEX 05, France. achille@mnhn.fr.

⁴ The Lewis B. and Dorothy Cullman Program for Molecular Systematics Studies, The New York Botanical Garden, Bronx, New York 10458-5126, U.S.A. tmotley@nybg.org.

⁵ Missouri Botanical Garden, P.O. Box 299, St. Louis, Missouri 63166-0299, U.S.A. pete.lowry@mobot.org.

known at the time. Schumann (1891) rejected Baillon's ideas and restricted the circumscription of *Guettarda*, excluding taxa usually placed in *Antirhea* and *Laugieria*. While Schumann's concept has been followed by most authors since (e.g., Standley, 1934; Bremekamp, 1966; Airy Shaw, 1973; Steyermark, 1972, 1974; Robbrecht, 1988), a modern comprehensive review of the genus is lacking.

Guettarda is currently regarded as a large, nearly pan-tropical genus of trees and shrubs. It is characterized by flowers with usually imbricate corolla lobes, 2- to 9(to 20)-locular fruits with a stony endocarp and one seed per locule, and a non-persistent calyx. In the Neotropics, *Guettarda* is thought to comprise anywhere from nearly 80 (Taylor et al., 2004) to more than 130 species (Andersson, 1992), and is morphologically quite heterogeneous, including taxa originally described or placed in several genera once regarded as distinct, e.g., *Dicrobotryum*, *Laugieria*, *Matthiola*, and *Tournefortiopsis*. Furthermore, the inclusion of *Antirhea* (or *Stenostomum*, to which Neotropical *Antirhea* species are currently assigned) can also be inferred based on the recent placement of the South American species *A. surinamensis* (described by Bremekamp, 1959) in synonymy under *Guettarda acreana* K. Krause by Taylor et al. (2001).

In the Paleotropics, a single, common, coastal species (*Guettarda speciosa* L.), which ranges from eastern Africa to Polynesia, accounts for most of *Guettarda*'s broad distribution. The other Old World species (ca. 25 in all) are almost exclusively narrow endemics on New Caledonia and comprise a morphologically diverse group. These were assigned to *Guettarda* by Baillon (1879) and Guillaumin (1948) in accordance with their broad concept of the genus, although they differ from its other members by several important characters including a dioecious sexual system and a persistent calyx. As currently circumscribed, the genus *Guettarda* thus has two main centers of diversity, the Neotropics and New Caledonia, showing an unusual trans-Pacific distribution shared with only a few other genera of Rubiaceae, such as *Augusta* Pohl sensu Kirkbride (1997) (including *Lindenia* Benth.) (Robbrecht, 1988).

Recent molecular phylogenetic studies have provided little evidence regarding the monophyly of *Guettarda*. Molecular sequence data for the genus are scarce, and, apart from the inclusion of a few species in family-level phylogenies, the only taxa studied to date are those used by Rova (1999) and Rova et al. (2002), who examined *rps16* and *trnL-F* sequences of a limited number of exemplar taxa. However, due to insufficient levels of sequence variation, trees based

on these markers place the six *Guettarda* species they sampled in a polytomy along with several allied genera, viz. *Chomelia*, *Malanea*, *Neolaugeria*, *Stenostomum* (referred to as *Antirhea*), and *Timonius*. Considering the wide range of interpretations (often contradictory) of relationships based on the morphological, biogeographical, and molecular data, it seems opportune to examine more closely the phylogenetic status of *Guettarda*, as currently defined, and its affinities with its apparent close allies.

Guettarda and the other genera currently included in Guettardeae (Table 1) are generally grouped together based on a set of common characters including: a woody habit, entire interpetiolar stipules (divided in *Neoblakea*), axillary inflorescences (terminal in *Dichilanthe*, *Machaonia*, and *Neoblakea*), a single, apically attached, pendulous ovule in each cell of the ovary, fleshy fruits with 2 to many unilocular pyrenes or a plurilocular endocarp or putamen (dry capsular fruits in *Machaonia*), and elongated seeds with poorly-developed endosperm (Robbrecht, 1988). Using this definition, the tribe contains perhaps 12 to 15 genera and more than 500 species, ranging in distribution from coastal East Africa to Polynesia and the Neotropics (Achille, unpublished data). The analysis of Rova et al. (2002) using sequence data from the chloroplast *trnL* intron and *trnL-F* spacer suggested a much broader circumscription of Guettardeae (Table 1) that includes several genera formerly placed in two other tribes, Isertiae and Rondeletiae. However, the expanded notion of Guettardeae proposed by Rova et al. (2002) seems problematic because the additional taxa they included have morphologies strikingly different from those of Guettardeae s. str., as reflected by the fact that these elements (whose distinctive features include strictly terminal inflorescences, mostly pluriovulate locules and capsular fruits, or sometimes sub-capsular fruits in *Gonzalagunia*) were placed by Robbrecht (1988) in subfamily Cinchonoidae, whereas Guettardeae were assigned to Antirheoideae.

Within Guettardeae (sensu Robbrecht), several elements appear to be most closely related to *Guettarda* (as currently circumscribed), together comprising core Guettardeae (as defined in Table 1): viz. *Antirhea* (including *Guettardella*), *Bobea*, *Chomelia*, *Guettarda*, *Malanea*, *Neolaugeria*, *Ottoschmidtia*, *Pittoniotis*, *Stenostomum*, and *Timonius*. These 10 genera form a morphologically homogeneous group whose recognition is consistent with available molecular data. In the phylogenies from Rova (1999) and Rova et al. (2002), the core Guettardeae they sampled (except *Bobea*) comprise a strongly supported monophyletic group, while the remainder of the tribe (sensu

Table 1. Genera included in Guettardeae by Robbrecht (1988) and in the “Guettardeae” s.l. clade in the phylogenies of Rova (1999) and/or Rova et al. (2002); those retained in core Guettardeae (as defined here) are indicated in the right column. excl. = excluded; n.s. = genus not studied.

Genus	Guettardeae sensu Robbrecht (1988)	Guettardeae clade of Rova (1999) & Rova et al. (2002)	Genera in core Guettardeae (as defined here)
<i>Allenanthus</i> Standl.	excl.	<i>Allenanthus</i>	
<i>Antirhea</i> Comm. ex Juss. ¹	<i>Antirhea</i>	n.s.	<i>Antirhea</i>
<i>Arachnothryx</i> Planch.	excl.	<i>Arachnothryx</i>	
<i>Bobea</i> Gaudich.	<i>Bobea</i>	excl.	<i>Bobea</i>
<i>Chomelia</i> Jacq.	<i>Chomelia</i>	<i>Chomelia</i> ²	<i>Chomelia</i>
<i>Cuatrecasasiodendron</i> Standl. & Steyerl.	excl.	<i>Cuatrecasasiodendron</i>	
<i>Dichilanthe</i> Thwaites	<i>Dichilanthe</i>	n.s.	
<i>Gonzalagunia</i> Ruiz & Pav.	excl.	<i>Gonzalagunia</i>	
<i>Guettarda</i> L.	<i>Guettarda</i>	<i>Guettarda</i> ²	<i>Guettarda</i>
<i>Guettardella</i> Champ. ex Benth.	<i>Guettardella</i>	n.s.	within <i>Antirhea</i>
<i>Javorkaea</i> Borhidi & Komlodi	excl.	<i>Javorkaea</i>	
<i>Machaonia</i> Bonpl.	<i>Machaonia</i>	<i>Machaonia</i>	
<i>Malanea</i> Aubl.	<i>Malanea</i>	<i>Malanea</i> ²	<i>Malanea</i>
<i>Neoblakea</i> Standl.	<i>Neoblakea</i>	<i>Neoblakea</i>	
<i>Neolaugeria</i> Nicolson	<i>Neolaugeria</i>	n.s.	<i>Neolaugeria</i>
<i>Ottoschmidtia</i> Urb.	<i>Ottoschmidtia</i>	n.s.	<i>Ottoschmidtia</i> (n.s.)
<i>Rogiera</i> Planch.	excl.	<i>Rogiera</i>	
<i>Pittoniotis</i> Griseb.	<i>Pittoniotis</i>	n.s.	<i>Pittoniotis</i>
<i>Stenostomum</i> C. F. Gaertn.	within <i>Antirhea</i>	<i>Antirhea</i> ^{2,3}	<i>Stenostomum</i>
<i>Timonius</i> DC.	<i>Timonius</i>	<i>Timonius</i> ²	<i>Timonius</i>

¹ Sensus Chaw & Darwin (1992).
² Genera assigned to the core Guettardeae clade by Rova (1999) and/or Rova et al. (2002).
³ The authors actually sampled only one species, *Antirhea acutata* (DC.) Urb., which is currently placed in *Stenostomum* (Borhidi & Fernandez, 1993–1994).

Robbrecht, 1988), except *Dichilanthe* (which was not sampled), are scattered among a paraphyletic group. Furthermore, core Guettardeae, as circumscribed here, which correspond to Baillon’s (1879, 1880) broadly defined *Guettarda*, display none of the distinctive features (mentioned above) that characterize *Dichilanthe*, *Neoblakea*, *Machaonia*, or the genera newly included in the tribe by Rova (1999) and Rova et al. (2002).
Although *Bobea* has always been included in Guettardeae, Rova (1999) suggests that it would have to be excluded in order to render the circumscription of the tribe (s.l.) monophyletic. Morphology, however, provides very strong support for the inclusion of *Bobea* within the core group of Guettardeae. *Bobea* is hardly distinguishable from *Timonius*, where it was placed in synonymy by Candolle (1830), and Baillon (1880) included both genera within *Guettarda* s.l. More recently, Darwin and Chaw (1990) regarded *Bobea* as distinct, but closely related to *Timonius* on the basis of morphology. In the field, individuals of *Bobea* are immediately recognizable as belonging to Guettardeae on the basis of fruit structure and overall morphology (pers. obs.). Notwithstanding Rova’s (1999) sugges-

tion, we have thus provisionally chosen to regard *Bobea* as a member of core Guettardeae.
Circumscriptions of the genera comprising core Guettardeae have varied widely from one treatment to another. Two genera, *Antirhea* and *Timonius*, in addition to *Guettarda* itself, play a central role in the group, as most species have at one time or another been associated with at least one of them. The Neotropical genus *Chomelia* (including *Anisomeris* C. Presl) comprises ca. 50 species and may be associated with *Guettarda* (Taylor et al., 2004), as the two are difficult to distinguish from one another on the basis of unequivocal characters. For example, Schumann (1891), Standley (1934), and Steyermark (1974) separated *Chomelia* from *Guettarda* on the basis of corolla aestivation (valvate vs. imbricate, respectively), but Burger and Taylor (1993) recognized both aestivation types in *Chomelia*. Furthermore, *trnL-F* sequence data, although limited, suggest that the two genera are related (Rova et al., 2002), as the only *Chomelia* sampled (*C. tenuifolia* Benth.) clusters with one of the five *Guettarda* species (*G. crispiflora* Vahl).
Antirhea (including *Guettardella*), *Neolaugeria*, *Stenostomum*, and *Pittoniotis* appear to comprise

a complex distributed in the Neotropics and the Indo-Pacific region, characterized by mostly 1 to 5 imbricate corolla lobes, 2 to 12 united pyrenes, and a persistent calyx. All the Paleotropical species in this group appear to be dioecious, a fact that was not discovered until recently (Chaw & Darwin, 1992). The monotypic, Neotropical genus *Pittoniotis*, described by Grisebach (1858), is readily distinguished by its long-exserted stamens, perfect flowers, and paniculate inflorescences. However, Hemsley (1881) transferred *Pittoniotis* to *Antirhea*, a decision that has been followed by some authors (Schumann, 1891; Dwyer, 1980). Hooker (1873) merged *Stenostomum*, a Caribbean genus, and *Guettardella*, a small South Asian genus, into *Antirhea*. Most subsequent authors have regarded *Stenostomum* and *Antirhea* as congeneric (e.g., Schumann, 1891; Standley, 1934; Bremekamp, 1966; Airy Shaw, 1973; Steyermark, 1974; Robbrecht, 1988). According to this broad generic circumscription, *Antirhea*, like *Guettarda*, exhibits an unusual trans-Pacific distribution.

Recently, Jansen (1981) restricted *Antirhea* to two species from the Mascarene Islands and Madagascar, and assigned all the other Old World species to *Guettardella*. His generic delimitations were based largely on the number of flowers per inflorescence, number of ovary cells, geographic distribution, and also an erroneous interpretation of floral sexuality. By contrast, Chaw and Darwin (1992) retained all the Old World species in *Antirhea* (36 species), within which they distinguished three subgenera, *A.* subg. *Antirhea*, *A.* subg. *Guettardella* (Champ. ex Benth.) Chaw, and *A.* subg. *Mesocarpa* Chaw, while tentatively suggesting that *Stenostomum* represents a distinct genus characterized by hermaphroditic, (4 to)5-merous flowers, ebracteolate inflorescences, and a Neotropical distribution. Following their suggestion, Borhidi and Fernandez (1993–1994) formally transferred the 39 American species of *Antirhea* to *Stenostomum*, and reduced *Neolaugeria* (4 to 6 Caribbean species, distinguished by the united stipules and the 4- to 6-locular ovary) to a section of *Stenostomum*. However, Moynihan and Watson (2001) rejected the inclusion of *Neolaugeria* in *Stenostomum* based on phylogenetic evidence from ITS sequence variation of three species of *Neolaugeria* and one of *Stenostomum*.

The large genus *Timonius* is traditionally characterized by having fruits with separate pyrenes, although this feature is also encountered in *Bobea*. *Timonius* comprises more than 180 species (Darwin, 1994) distributed from the Seychelles to Polynesia and from the Philippines to northern Australia. It is morphologically heterogeneous, but is classically interpreted to include all dioecious Guettardeae with

valvate corolla lobes and separate pyrenes. *Bobea* comprises four species endemic to Hawaii (Darwin & Chaw, 1990), which differ mostly from *Timonius* by the presence of imbricate corolla lobes.

Finally, two Neotropical genera, *Ottoschmidtia* and *Malanea*, stand out because their circumscriptions have rarely been questioned. *Ottoschmidtia* (3 species) is easily separated from other Guettardeae by its 2-locular ovary and zygomorphic corollas. *Malanea*, with ca. 35 species (Taylor et al., 2004), is recognized by its twining habit, paniculate to spiciform inflorescence, exserted stamens, and stipules with a distinctive morphology, although Taylor et al. (2004) regard the distinction between *Malanea* and *Chomelia* as unclear.

In an effort to evaluate the phylogenetic status of *Guettarda*, as currently defined, we have endeavored to include a representative group of species belonging to the genera of core Guettardeae. Our study employs DNA sequences from the internal transcribed spacer region (ITS, spacer 1 & 2, and 5.8S gene between the nuclear ribosomal DNA 18S–26S genes). Using a broad sampling of 21 species of *Guettarda* (as currently circumscribed) and 21 species belonging to 10 other genera of Guettardeae (sensu Rova, 1999), we thus (1) evaluate the monophyly of *Guettarda*; (2) provide some insights regarding the phylogenetic status of other core genera in relation to *Guettarda*, especially those whose circumscriptions have varied widely; and (3) attempt to identify morphological characters that might prove valuable for further taxonomic and phylogenetic studies within Guettardeae. We have also examined the geographic distributions of *Guettarda* and *Antirhea* in light of the results of our phylogenetic analysis, as these two genera are among the only Rubiaceae to exhibit a disjunct, nearly pantropical distribution.

MATERIALS AND METHODS

TAXON SAMPLING

To ensure the best possible representation of infrageneric groups within *Guettarda*, for which no comprehensive infrageneric treatment is available, we delimited informal morphological groups on the basis of examination of herbarium material at P and NY, field observations made by the first author in New Caledonia, and information gathered from the literature (in particular Howard, 1989; Standley, 1934; Standley & Williams, 1975). Furthermore, we attempted to include species from various phytogeographical subdivisions of the Neotropical regions, as defined by Andersson (1992). We have also sought to sample, as exhaustively as possible, at the generic

(and sometimes infrageneric) level within the other core genera of Guettardeae. To delimit infrageneric groups, we consulted the treatments of *Antirhea* by Chaw and Darwin (1992) and *Stenostomum* by Borhidi and Fernandez (1993–1994), and the partial revisions of *Timonius* by Darwin (1993, 1994), supplemented by informal morphological groups that were identified in the same manner as *Guettarda* (Valeton, 1909; Wong, 1988).

Special attention was given to including the type species of each genus, and, when possible, a minimum of 2 species were sequenced per genus, subgenus, or informal group. The 42 taxa used in the analysis represent 9 of the 10 core genera of Guettardeae (Table 2), including the type species of 8 currently recognized genera (*Guettarda speciosa*, *Antirhea borbonica*, *Bobea elatior*, *Chomelia spinosa*, *Neolaugeria resinosa*, *Pittoniotis trichantha*, *Stenostomum lucidum*, and *Timonius timon*) and 3 genera currently placed in synonymy, namely, *Dicrobotryum*, *Guettardella*, and *Matthiola* (typified by *Guettarda divaricata*, *Antirhea chinensis*, and *Guettarda scabra*, respectively). Because of a lack of material, *Ottoschmidtia* could not be included, and a single species each was sequenced for *Chomelia* and *Malanea*. For *Antirhea* and *Timonius*, we included 4 and 5 species, respectively, which were selected to represent as much morphological diversity as possible. Three previously published sequences were used for *Neolaugeria densiflora*, *Stenostomum myrtifolium*, and *Timonius nitidus* (Moynihan & Watson, 2001), while 39 new Guettardeae sequences were generated. Two outgroup taxa (*Cuatrecasasi dendron spectabile* and *Gonzalagunia rosea*) were selected from Guettardeae sensu Rova, since they are both placed near to the genera of core Guettardeae in his phylogenies (Rova, 1999).

DNA EXTRACTION

Leaf samples were collected either in silica gel or from herbarium sheets. Genomic DNA was extracted from approximately 1 cm² of dried leaf tissue using a modified CTAB methodology. Leaf material was ground in a lysing matrix "A" tube (Qbiogene, Carlsbad, CA) and pulverized for 15 sec. in a Fastprep machine FP-120 (Qbiogene) bead mill at speed 5. Subsequently, 500 µl of Carlson Lysis Buffer (2 g CTAB, 8.18 g NaCl, 0.745 g EDTA, 10 ml 1 M Tris/HCl pH 7.0, nanopure water to 100 ml, verified to pH 9.5, autoclaved, then 1 g PEG 4000 added when cool) and 75 µl of β-mercaptoethanol were added to each tube and incubated at 74°C with occasional shaking for 60–90 min. Following incubation, 575 µl of SEVAG (24 : 1 chloroform : isoamyl alcohol) were added to each tube; the tubes were placed on a tipping

board for 30 min. at room temperature, and then centrifuged at 14,000 rpm for 1 min., and ~350 µl of supernatant was removed and added to new tubes containing 1050 µl of NaI solution, 20 µl Glassmilk, and 4 µl TBE modifier (Qbiogene). The tubes were placed on a tipping board for 30 min. at room temperature. Afterward, the tubes were centrifuged at 14,000 rpm for 1 min. and all of the supernatant was discarded. Next, each Glassmilk pellet was washed three times with 800 µl and once with 150 µl of ice cold New Wash solution (Qbiogene). After the final wash, all of the New Wash was aspirated from the Glassmilk pellet and 50 µl of 10 mM Tris-Cl (pH 8.5) elution buffer were added to resuspend the DNA. The tubes were incubated at ~55°C for ~10 min. and then centrifuged for 1 min. at 14,000 rpm. The supernatant containing the DNA was removed and transferred to new tubes and stored at –20°C.

DNA AMPLIFICATION

For DNA amplification, PCR was performed in a 25 µl mixture consisting of 2.5 µl 10× buffer with MgCl₂ (Perkin Elmer, Foster City, CA), 9.3 µl autoclaved nanopure water, 2.5 µl BSA (bovine serum albumin), 2.5 µl dNTP, 1 µl each of two 20 µM primers, 5 µl betaine, 0.2 µl Taq polymerase (Qiagen, Valencia, CA), and 1 µl of genomic DNA. All PCR and cycle sequencing reactions were run on a Gene Amp PCR system 9600 (Applied Biosystems, Foster City, CA). The ITS region was amplified using primers NY183 (5'-CCTTATCATTAAGAGGAAGGAG-3') and NY43 (5'-TATGCTTAAAYTCAGCGGGT-3'), and when necessary two additional internal primers were employed, ITS2 (5'-GCTACGTTCTTCATC-GATGC-3') and ITS3b (5'-GCATCGATGAAGAACG-TAGC-3') (White et al., 1990; Baldwin, 1992; Baum et al., 1998). The PCR conditions for amplification of the ITS region were: 1 cycle of 97°C for 50 sec.; 30 cycles of 97°C for 50 sec., 53°C for 50 sec.; and 1 cycle of 72°C for 1 min. 50 sec., hold 72°C for 7 min., hold at 4°C.

DNA SEQUENCING

To detect successfully amplified products, as well as the possible presence of multiple ITS paralogues of varying length (as discussed by Alvarez & Wendel, 2003) and possible contamination of negative controls, PCR products were examined on 1% agarose gels stained with ethidium bromide and visualized under ultraviolet light. Amplified products were purified with spin columns from the QIAquick PCR purification kit (Qiagen) following protocols provided

Table 2. Species, origin, and voucher information for the 42 ITS sequences used in the present study. Voucher information, with collections italicized, is given for new sequences; literature citation is provided for previously published sequences of three taxa. Herbarium acronyms follow *Index Herbariorum* (Holmgren et al., 1990). Partial sequences are indicated in parentheses following GenBank accession number.

Species	Origin	Voucher information / literature citation	GenBank accession
<i>Antirhea borbonica</i> J. F. Gmel.	Madagascar	<i>Rakotomalaza 1248</i> (P)	DQ063666
<i>Antirhea chinensis</i> (Champ. ex Benth.) F. B. Forbes & Hemsl.	China	<i>K. Y. Tam s.n.</i> , Hong Kong (P)	DQ063702
<i>Antirhea rhamnoides</i> (Baill.) Chaw	New Caledonia	<i>Achille 688</i> (P)	DQ063686
<i>Antirhea smithii</i> (Fosberg) Merr. & L. M. Perry	Fiji	<i>A. C. Smith 7685</i> (NY)	DQ063667(ITS1)
<i>Bobea elatior</i> Gaudich.	Hawaii	<i>Motley 2536</i> (NY)	DQ063668
<i>Bobea sandwicensis</i> (A. Gray) Hillebr.	Hawaii	<i>Takeuchi 3302</i> (NY)	DQ063669
<i>Chomelia spinosa</i> Jacq.	Costa Rica	<i>Seigler 12306</i> (NY)	DQ063703(ITS1)
			DQ063704(ITS2)
<i>Cuatrecasasi dendron spectabile</i> Steyererm.	Colombia	<i>Rova et al. 2093</i> (GB)	DQ063670
<i>Gonzalagunia rosea</i> Standl.	Costa Rica	<i>Achille s.n.</i> , Cult. NYBG (P)	DQ063671
<i>Guettarda acreana</i> K. Krause	French Guiana	<i>Mori 18842</i> (NY)	DQ063672
<i>Guettarda combsii</i> Urb.	Belize	<i>Lentz et al. 2853</i> (NY)	DQ063673
<i>Guettarda crispiflora</i> Vahl	Colombia	<i>Andersson et al. 2088</i> (GB)	DQ063674
<i>Guettarda divaricata</i> (Humb. & Bonpl.) Standl.	Guyana	<i>Jansen 4908</i> (NY)	DQ063675
<i>Guettarda elliptica</i> Sw.	Puerto Rico	<i>Axelrod 3060</i> (NY)	DQ063676
<i>Guettarda heterosepala</i> Guillaumin	New Caledonia	<i>Achille 697</i> (P)	DQ063677
<i>Guettarda hirsuta</i> (Ruiz & Pav.) Pers.	Ecuador	<i>Delprete 6054</i> (NY)	DQ063678(ITS1)
<i>Guettarda humboldtensis</i> Guillaumin	New Caledonia	<i>Achille 672</i> (P)	DQ063679
<i>Guettarda krugii</i> Urb.	Puerto Rico	<i>Struwe 1102</i> (NY)	DQ063680
<i>Guettarda macrosperma</i> Donn. Sm.	Costa Rica	<i>Hammel 19883</i> (NY)	DQ063681
<i>Guettarda nannocarpa</i> Urb. & Ekman	West Indies	<i>Liogier 16631</i> (NY)	DQ063682
<i>Guettarda noumeana</i> Baill.	New Caledonia	<i>Achille 662</i> (P)	DQ063683
<i>Guettarda pohliana</i> Müll. Arg.	Bolivia	<i>Vee 40536</i> (NY)	DQ063684
<i>Guettarda pungens</i> Urb.	Puerto Rico	<i>Grimes 3261</i> (NY)	DQ063685
<i>Guettarda scabra</i> (L.) Lam.	Puerto Rico	<i>Aceredo Rodriguez 2840</i> (NY)	DQ063687
<i>Guettarda</i> sp. "Cuba"	Cuba	<i>Areces 6538</i> (NY)	DQ063688
<i>Guettarda speciosa</i> L.	New Caledonia	<i>Achille 661</i> (P)	DQ063689
<i>Guettarda splendens</i> Baill.	New Caledonia	<i>Achille 1010</i> (P)	DQ063690
<i>Guettarda stenophylla</i> Urb.	West Indies	<i>Liogier 16950</i> (NY)	DQ063705(ITS1)
			DQ063706(ITS2)
<i>Guettarda trimera</i> Guillaumin	New Caledonia	<i>Achille 870</i> (P)	DQ063691
<i>Guettarda uruquensis</i> Cham. & Schltdl.	Brazil	<i>HU CS 12227</i> (NY)	DQ063692
<i>Malanea macrophylla</i> Bartl. ex Griseb.	Guyana	<i>Jansen-Jacobs 5396</i> (NY)	DQ063693
<i>Neolaugeria densiflora</i> (Griseb.) Nicolson	Bahamas	Moynihan & Watson (2001)	AF323055(ITS1)
			AF323056(ITS2)
<i>Neolaugeria resinosa</i> (Vahl) Nicolson	Puerto Rico	<i>Taylor & Gereau 10501</i> (NY)	DQ063694
<i>Pittoniopsis trichantha</i> Griseb.	Panama	<i>Croat 9311</i> (NY)	DQ063695(ITS1)
<i>Stenostomum acutatum</i> DC.	Puerto Rico	<i>Axelrod 3288</i> (NY)	DQ063696
<i>Stenostomum lucidum</i> (Sw.) C. F. Gaertn.	Dominican Republic	<i>Aceredo Rodriguez 8468</i> (NY)	DQ063697
<i>Stenostomum myrtifolium</i> Griseb.	Bahamas	Moynihan & Watson (2001)	AF323059(ITS1)
			AF323060(ITS2)
<i>Timonius densiflorus</i> Valetton	Papua New Guinea	<i>Takeuchi 8689</i> (NY)	DQ063698
<i>Timonius flavescens</i> Baker	Malaysia	<i>Laman et al. 18</i> (NY)	DQ063699
<i>Timonius nitidus</i> Fern.-Vill.	Guam	Moynihan & Watson (2001)	AF323063(ITS1)
			AF323064(ITS2)
<i>Timonius polygamus</i> (Forst. f.) Robinson	French Polynesia	<i>Motley 2044</i> (NY)	DQ063700
<i>Timonius timon</i> (Spreng.) Merr.	Papua New Guinea	<i>Motley 2309</i> (NY)	DQ063701

by the manufacturer. Purified products were cycle sequenced with dye terminator ABI Prism® Ready reaction mix (Applied Biosystems) using dRhodamine or Big Dye v1.0 (1/8 reaction) and 5% dimethyl sulfoxide. Primers for cycle sequencing were the same as those used in the PCRs. Cycle sequencing conditions were: 1 cycle of 95°C for 1 min.; 32 cycles of 96°C for 10 sec., 50°C for 5 sec., 60°C for 3 min.; and hold at 4°C. Products were purified using hydrated Sephadex G-50 DNA Grade F columns (Amersham Pharmacia Biotech, Piscataway, NJ) and dehydrated in a speed vac. The DNA was resuspended in 2.2 µl of formamide (83.5%) and EDTA blue-dextran loading dye (16.5%), heated at 95°C for 2 min., and immediately placed on ice. Sequences were run on a polyacrylamide gel on an ABI Prism 377 DNA sequencer (Applied Biosystems). Gels consisted of 18 g urea ultrapure grade (Amresco, Solon, OH), 0.5 g Amberlite mixed bed resin MB-150 (Sigma-Aldrich, Milwaukee, WI), 27 ml nanopure water, and 5.6 ml Acryl/Bis (Amresco).

PHYLOGENETIC ANALYSIS

Sequences were edited and aligned in Sequencher version 3.1.2 (Gene Codes Corp., Ann Arbor, MI) followed by manual optimization. Gaps were inserted to keep the number of informative characters (indels and substitutions) to a minimum. Indels were treated as unordered, multistate characters; those of equal length that occurred in more than one sequence were considered homologous and scored as separate binary characters added to the data matrix. Insertions that had slightly different sequences in two or more taxa were treated as homologous (Barriel, 1994), and the variable bases within these sequences were then recoded as new characters (and treated as missing for the taxa in which the insertions were absent). The data matrix is available from the first author.

A heuristic parsimony search was performed using PAUP* version 4.01b (Swofford, 1998), with 100 random addition replicates, TBR branch swapping, and MULPARS option in effect. Relative levels of homoplasy and synapomorphy in the data set were calculated using the consistency index (CI), the retention index (RI), and the rescaled consistency index (RC), as implemented in PAUP*. Internal branch support was estimated with bootstrap analysis (Felsenstein, 1985) using 1000 replicates with TBR branch swapping and simple taxon addition. Pairwise sequence divergence between species of core Guettardeae was calculated with PAUP* ("p" distance, excluding ambiguously aligned sites).

A Bayesian inference of phylogeny (BI) was obtained with the program MrBayes 3.0b4 (Huelsen-

beck & Ronquist, 2001) using the GTR+I+Γ model of nucleotide substitution, a general time reversible model that allows (1) a proportion of invariable sites and (2) among-site rate heterogeneity following a gamma distribution. This model was selected as the best fit model for our data from a comparison of 24 models using the Akaike information criterion (Akaike, 1974) as implemented in MrModeltest 2.2 (Nylander, 2004), a modified version of Modeltest 3.6 (Posada & Crandall, 1998). The analysis of the ITS data set (excluding indels) was run using 2×10^6 generations, which were performed on four Markov chains (the temperature of the chains and other parameters were left at default values) with trees sampled every 100 generations. Markov chain convergence occurred rapidly (before 2×10^4 generations), and 2000 trees (2×10^5 generations) were discarded as the "burn-in" of the process. The remaining trees were then used to construct a 50% majority rule consensus tree, which provides estimations of each clade's posterior probability. As a control (Huelsenbeck et al., 2002), four independent searches were run, which yielded similar results. All saved trees from the independent runs ("burn-ins" excluded) were pooled to obtain a consensus tree. Unlike bootstrap support values, posterior probabilities are interpreted as true probabilities for each clade under the assumed model (Rannala & Yang, 1996).

RESULTS

Direct sequencing of the purified PCR products yielded single bands on agarose gels and unambiguous ITS sequences (without obvious multiple bases), showing no evidence of paralogs of varying length. The length of the sequences (including ITS1 + 5.8S + ITS2) ranged from 590 base pairs (bp) (in *Guettarda stenophylla*) to 609 bp (in *Gonzalagunia rosea*). The aligned matrix comprises 680 positions (655 of which represent the aligned sequences and 25 the coding of the indels, including autapomorphies). A total of 84 sites were excluded from the analysis because their alignment was uncertain (most of the indels present among these 84 positions were, however, retained in their recoded form as indicated above). Of these 596 characters included in the analysis, 421 were constant, 76 variable but uninformative, and 99 potentially informative. For two species (*Guettarda hirsuta* and *Pittoniotis trichantha*), ITS2 sequences could not be obtained. *Chomelia spinosa* and *G. stenophylla* could not be sequenced for a region in the 5.8S cistron (17 and 19 bp long, respectively), and the end of ITS2 could not be sequenced for *Timonius flavescens* (46 missing bp). In these cases, the bases were treated as missing data.

The 5.8S cistron is 165 bp in length for all taxa, except in the sequence of *Stenostomum myrtifolium* provided by Moynihan and Watson (2001), which is 2 bp shorter. As often observed (e.g., by Andreasen et al., 1999; Moynihan & Watson, 2001), few mutations occur within this region. The ITS 1 and 2 regions in the 40 species of core Guettardeae included in our sample, range in length from 213 to 223 and 207 to 224 bases, respectively. These lengths fall within the range reported for other angiosperms (Baldwin et al., 1995) and for other groups of Rubiaceae (McDowell & Bremer, 1998; Andreasen et al., 1999; Persson, 2000; Razafimandimbison & Bremer, 2002). Percent pairwise distances between core Guettardeae species range from 0 to 14.2 for ITS 1 (mean = 7.6 ± 2.4), 0 to 13.0 for ITS 2 (mean = 5.3 ± 2.1), and 0 to 9.8 (mean = 5.0 ± 1.5) for the whole ITS region. This amount of ITS variation is comparable to levels usually reported for a single genus (Baldwin et al., 1995), such as *Viburnum* L. (Donoghue & Baldwin, 1993; reviewed in Baldwin et al., 1995) or infrageneric taxa such as *Gilia* sect. *Giliandra* A. Gray (Porter, 1993; reviewed in Baldwin et al., 1995). Compared to other groups of Rubiaceae, variation within core Guettardeae (ca. 500 species) is similar to (or in the case of ITS1, lower than) that reported by McDowell and Bremer (1998) for *Exostema* (Pers.) Rich. ex Humb. & Bonpl. (25 species), and similar to the level observed by Nepokroeff et al. (1999) within the main lineages of tribe Psychotriaceae, but much lower than for the tribe as a whole (1000 to 1650 species).

The MP analysis yielded 1293 most parsimonious trees, each with a length of 445 (CI = 0.577, RI = 0.695, RC = 0.401). One of the shortest trees is given in Figure 1. The strict consensus (Fig. 2) is well resolved, although numerous clades are not significantly supported in the bootstrap (BS) analysis (BS < 50%). The ingroup node has strong support (BS = 99%) and the topology at the base of the ingroup is fully resolved but poorly supported, in contrast to the terminal branches. Eight pairs of species (*Guettarda divaricata*–*G. elliptica*, *G. nannocarpa*–*G. stenophylla*, *G. krugii*–*G. sp.* “Cuba,” *Stenostomum acutatum*–*S. myrtifolium*, *Bobea elatior*–*B. sandwicensis*, *G. crispiflora*–*G. hirsuta*, *Timonius nitidus*–*T. polygamus*, *Neolaugeria densiflora*–*N. resinosa*) receive high bootstrap support, ranging from 94 to 100%. *Pittoniotis* is sister to the rest of the ingroup, and the remainder of the genera comprise two main clades. The first of these clades contains most species of *Guettarda*, the majority of the Neotropical genera, and the Hawaiian endemic genus *Bobea*. Within this clade, two *Guettarda* species appear isolated as sisters of *Bobea* (comprising the “*Bobea* clade”). The second major clade comprises all the Paleotropical genera,

some *Guettarda* species (mostly from New Caledonia), and the West Indian taxa *Neolaugeria* and *Stenostomum* sect. *Stenostomum*. Within this second main clade, a weakly-supported Paleotropical dioecious subclade (BS = 63%) unites all the Paleotropical species included in the analysis except one (*Guettarda speciosa*), while the Neotropical subclades (the first comprising *Neolaugeria* and the second with *Stenostomum* sect. *Stenostomum* + *Guettarda acreana*) are paraphyletic at the base of the clade (no BS support).

Guettarda appears to be polyphyletic in our trees. A majority of the Neotropical taxa sampled are associated with the Paleotropical type species, *G. speciosa*, and thus comprise the “*Guettarda* s. str.” clade (BS = 57%). The three remaining species that occur in the New World are isolated (one is associated with *Stenostomum lucidum*, while the other two are part of the *Bobea* Clade) (Fig. 1). All the other *Guettarda* species sampled occur in the Old World and are included within a Paleotropical Dioecious Clade. The complexes of genera usually associated with the three principal members of core Guettardeae (*Guettarda*, *Antirhea*, and *Timonius*) are not evident in our trees. Our limited sampling suggests that *Timonius* and *Bobea* are monophyletic and, although often considered as close relatives, are placed in very distant clades in our trees. *Antirhea* + *Stenostomum* (= *Antirhea* s.l.) is polyphyletic, with *Antirhea* species forming a paraphyletic group with respect to *Timonius* and the New Caledonian *Guettarda* species in the Paleotropical Dioecious Clade, while the members of *Stenostomum* are represented in two additional clades, suggesting it, too, may be polyphyletic.

The BI tree is given in the right half of Figure 2. Its overall topology is quite similar to that of the MP tree and the two analyses essentially recover the same major clades. More clades are well supported in the BI analysis (with posterior probabilities exceeding 0.95) than in the MP analysis (some of which have BS values below 50%), but those clades that are well supported in the MP analysis are likewise in the BI tree. For example, the clade comprising New Caledonian *Guettarda* and 3 *Antirhea* species is strongly supported in the BI analysis (posterior probability, $P = 0.99$), but unsupported in the MP analysis (BS < 50%), whereas the clade comprising *G. divaricata* and three other Neotropical *Guettarda* species has strong support in both MP and BI analyses (BS = 97%, $P = 1.00$). The BI tree is slightly less resolved than the MP consensus tree. *Neolaugeria* appears sister to the rest of the ingroup, which forms a polytomy of three clades. The first of these clades encompasses most of the Neotropical genera, including *Guettarda* s. str., *Pittoniotis* and *Malanea* ($P = 0.57$); the second comprises the *Bobea* Clade ($P =$

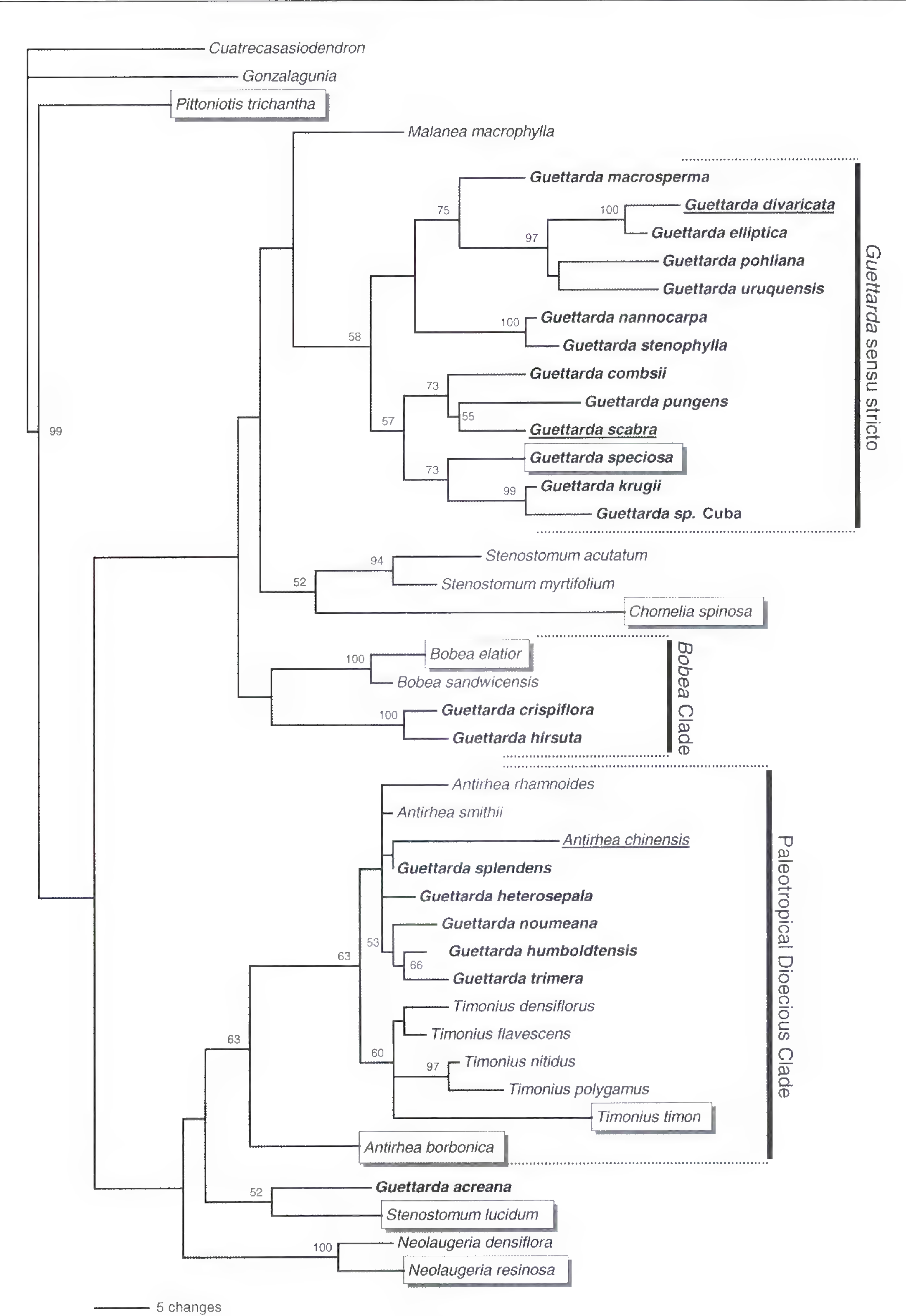


Figure 1. Phylogram of one of the 1293 most parsimonious trees (length = 445; CI = 0.577; RI = 0.695) resulting from the MP analysis of ITS data. Numbers on internodes indicate bootstrap support (in %). *Guettarda* species are in **bold**. Type species of currently recognized genera are placed in a box; type species of genera currently placed in synonymy are underlined (see text for details).

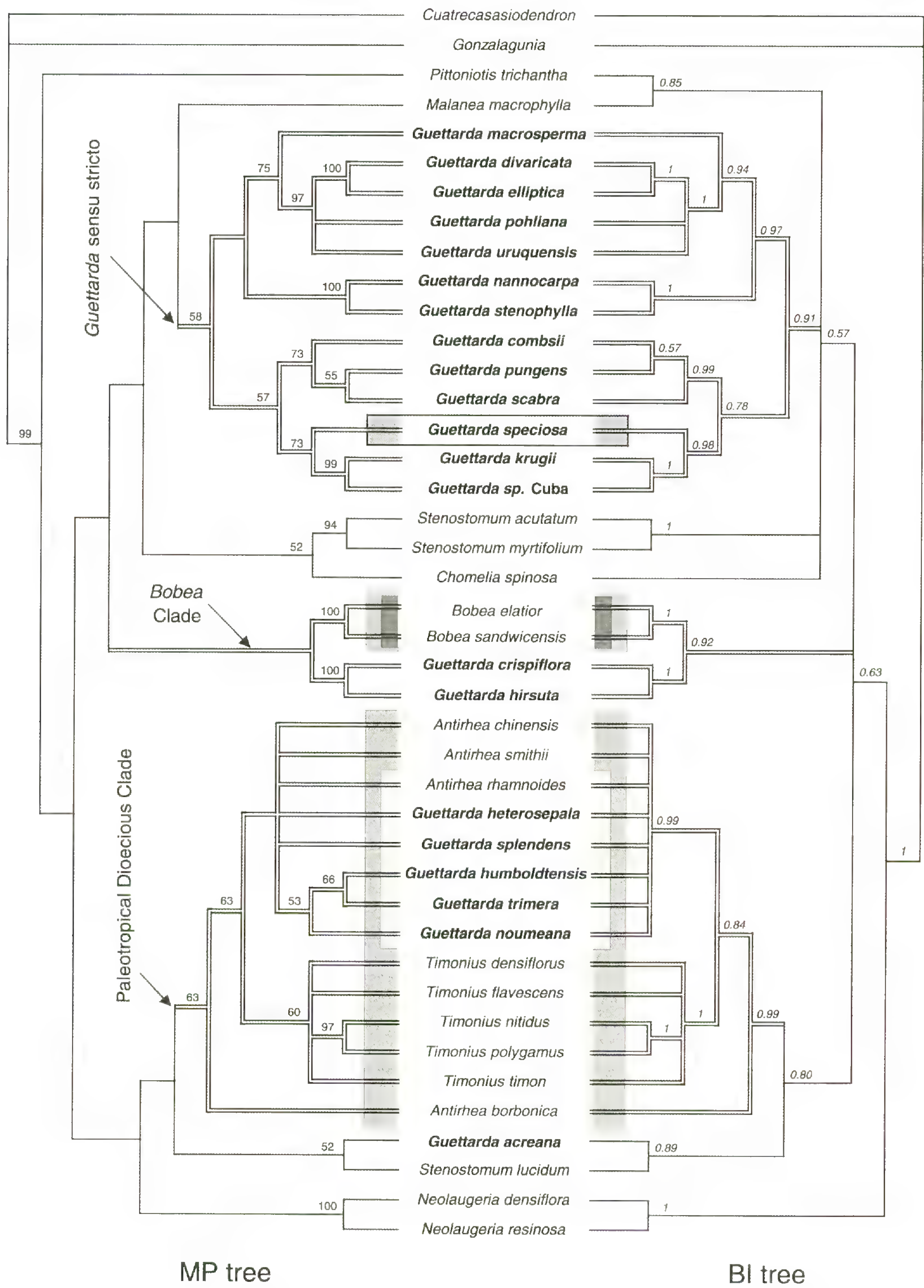


Figure 2. Comparison of the consensus trees from the MP and BI analyses of ITS data. The tree on the left is the strict consensus of 1293 most parsimonious trees (numbers on internodes indicate bootstrap values over 50%). The tree on the right is the 50% majority rule Bayesian consensus tree under the GTR+I+ Γ model of substitution from four 2×10^6 generations runs (numbers on internodes indicate posterior probabilities). *Guettarda* species are in **bold**. Clades discussed in the text are indicated by double lines. Geographic distribution of terminal taxa is indicated as follows: gray = paleotropical; dark gray = Hawaii endemic; light gray = endemic to New Caledonia; all others are Neotropical.

0.92); and the third clade ($P = 0.80$), which is also recovered in the MP analysis, includes the type of *Stenostomum* (*S. lucidum*), *Guettarda acreana*, and the well-supported Paleotropical Dioecious Clade ($P = 0.99$). Most of the smaller clades recovered in the BI tree are also found in the MP tree, but the position of *Pittoniotis* is inconsistent between the two analyses.

DISCUSSION

The poor support of the larger clades identified in the MP analysis (most with $BS < 50\%$ and none exceeding 63%) is unusual as ITS data are generally most informative at suprageneric levels (Baldwin et al., 1995). For example, in a study of Vanguerieae (Lantz et al., 2002), a similar-sized rubiaceaceous tribe (19 genera, ca. 600 species) that was examined using a comparable data set of 41 ingroup taxa, the major clades are both well-resolved and strongly supported. The situation found in Guettardeae, with low support and/or resolution among the internal nodes, has also been observed in several other groups of Rubiaceae, such as *Alibertia* A. Rich. ex DC. (Persson, 2000) and the Catesbaeeae-Chiococceae complex (Motley et al., 2005), as well as among groups of Asteraceae (Baldwin & Sanderson, 1998; Garcia-Jacas et al., 2001), Fabaceae (Allan & Porter, 2000), and Apiales (Plunkett & Lowry, 2001; Chandler & Plunkett, 2004; Plunkett et al., 2004). This pattern would be consistent with a hypothesized rapid radiation early in a group's evolution, and could explain the lack of support at the base of our trees, which stands in contrast to the morphological diversity that has served to segregate the core genera within the tribe.

POLYPHYLY OF GUETTARDA

Based on our analysis of ITS sequence data, *Guettarda*, as currently circumscribed, is clearly polyphyletic, with species distributed among four distinct clades (Fig. 1). Most of the Neotropical species belong to the first of these, the *Guettarda* s. str. clade ($BS = 58\%$, $P = 0.91$), which includes the type species, *G. speciosa*, from the Indo-Pacific region. The topology within this clade is well resolved (Fig. 2) even though the species are morphologically quite similar to one another. Members of this group are characterized by usually globose fruits with more than 2 locules, dichasial cymose inflorescences (sometimes reduced to a solitary flower), and a caducous calyx with a circular abscission scar (Fig. 3). These last two characters, although highly distinctive of *Guettarda* s. str., also occur (although not in combination) in three New Caledonian *Guettarda* species, where they may have evolved independently from the core Neotropical

lineage: *G. noumeana* and an unnamed close ally (not sampled) have a dichasial cymose inflorescence structure, and a recently discovered undescribed species (not sampled) has a dehiscent calyx with circular abscission.

Within the *Guettarda* s. str. clade, there are three main subclades. The first comprises species from Continental America ($BS = 75\%$, $P = 0.94$), a few of which, such as *G. elliptica*, extend slightly into the Caribbean Islands. *Guettarda divaricata*, the type species of *Dicrobotryum* Willd. ex Roem. & Schult., is part of this clade. Members of this group are characterized by small- to medium-sized, membranaceous leaves, and possibly also by their pollen (Fig. 2), which is similar to that found in *Antirhea* s. str. (oblate-spheroidal 3-colporate pollen with reticulate exine) (Achille, unpublished data). The second subclade includes two Caribbean species ($BS = 100\%$, $P = 1.00$), *G. nannocarpa* and *G. stenophylla*, with very small membranaceous leaves, solitary flowers, and small fruits. The third subclade, while weakly supported ($BS = 57\%$, $P = 0.78$), unites a group of morphologically distinct species mainly from the West Indies along with the Paleotropical *G. speciosa*, the type species. *Guettarda scabra*, the type of *Matthiola* (widely regarded as a generic synonym since Lamarck, 1792), also belongs to this group and closely resembles *G. speciosa*. The fruits of taxa in this third subclade are rather large, as are the leaves, which are often coriaceous or bullate and have scalariform venation. *Guettarda pungens* stands out within this group in having small, spiny leaves, and small fruits. However, all members of the third subclade share a distinct pollen type, characterized by an oblate-spheroidal shape, 3 to 4 pororate apertures, a perforated to reticulate tectum, and short columellae (Achille, unpublished data).

The inclusion of the widely distributed Indo-Pacific species *Guettarda speciosa* in an otherwise strictly Neotropical clade is perhaps best explained by relatively recent long-distance dispersal. *Guettarda speciosa* occurs in strand vegetation and coastal forests on coral limestone, and its fruits have air-filled cavities, making them well adapted to transport by ocean currents. This species is not found in Hawaii or the eastern Pacific, although this may simply reflect the random nature of dispersal. It is perhaps more surprising to note that *G. speciosa* appears to be most closely related to species from the West Indies (such as the widespread coastal species *G. scabra* mentioned above) rather than from the Pacific coast of the Americas, as one might expect.

The second clade containing members of *Guettarda* includes two Neotropical species (*G. crispiflora* and *G. hirsuta*) that are sister in our analysis to *Bohea* ($BS <$

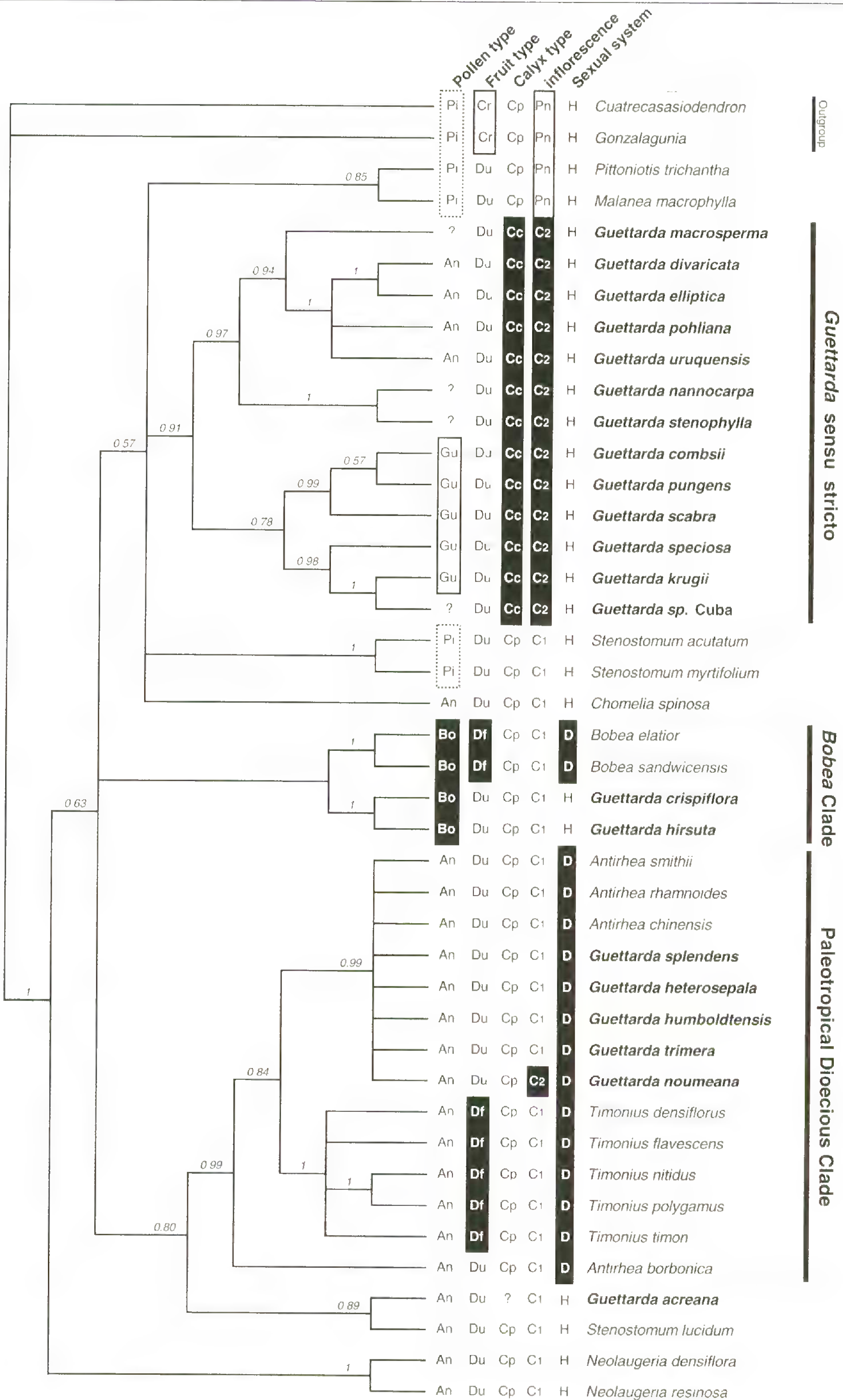


Figure 3. Important morphological characters mapped on the 50% majority rule Bayesian consensus tree. Numbers on internodes indicate posterior probabilities. *Guettarda* species are in **bold**. The symbols indicate the state for each category of characters (Pollen type: Pi = *Pittoniotis*; An = *Antirhea*; Gu = *Guettarda*; Bo = *Bobea*; ? = unknown. Fruit type: Cr = capsular; Du = drupaceous with united pyrenes; Df = drupaceous with free pyrenes. Calyx type: Cc = caducous by abscission; Cp = persistent on fruit; ? = variable. Inflorescence type: Pn = panicle; C2 = fundamentally dichasial cyme; C1 = fundamentally monochasial cyme. Sexual system: H = hermaphroditism; D = dioecy).

50%, $P = 0.92$). These taxa exhibit several distinctive characters that separate them from other members of *Guettarda*. Along with a few other apparently closely related species from Central and South America that were not sampled, they form a well-defined group characterized by large stipules with membranaceous margins, scalariform venation, bifid monochasial inflorescences (which differ from the dichasial cymes found in other Neotropical *Guettarda*), small 4-ribbed ovoid fruits with a minute and persistent calyx (see also species descriptions in, e.g., Standley, 1934; Steyermark, 1972; Taylor et al., 2004), as well as “*Bobea* type” pollen (see below under *Bobea*). Although additional molecular data will be required to confirm whether these taxa and *Bobea* are closely related, their distinctive morphological characters suggest that recognition at the generic rank might be in order, in which case the name *Tournefortiopsis* would be available, as it is typified by *T. reticulata* Rusby. (= *Guettarda tournefortiopsis* (Rusby) Standl.), a close relative of *G. crispiflora*.

The *Guettarda* species from New Caledonia constitute a third clade (Fig. 2) that is very well supported in the BI analysis ($P = 0.99$) although unsupported in the MP analysis (BS < 50%). This clade includes three species of *Antirhea* (*A. chinensis*, *A. rhamnoides*, and *A. smithii*) that would either belong to the segregate genus *Guettardella* according to Jansen (1984) or to *Antirhea* subg. *Guettardella* and *Antirhea* subg. *Mesocarpa* according to Chaw and Darwin (1992). This clade is nested within a broader Paleotropical Dioecious Clade (see below), and is the sister group of *Timonius*. The New Caledonian species of *Guettarda* are morphologically very distinct from the Neotropical members of the genus and from *G. speciosa*. In fact, several characters, including dioecism, a calyx that is almost always persistent in fruit, and monochasial cymes (Fig. 3), are reminiscent of *Antirhea* s.l. The placement of these species within the Paleotropical Dioecious Clade is thus consistent with morphology, and their inclusion in a broadly defined *Guettarda* by Baillon (1879), Guillaumin (1948), and more recently Jaffré et al. (2004) merely reflects an out-of-date concept of the genus that has been abandoned in most other parts of the world. It therefore would seem appropriate to exclude the New Caledonian species from *Guettarda* and transfer them to another genus (see discussion below under *Antirhea*).

It is worth noting that the resolution within the New Caledonian *Guettarda* clade is very weak despite the fact that its members are morphologically quite distinct from one another. Many New Caledonian species of *Guettarda* show one or more characters that are rare or unknown elsewhere in Guettardeae, such

as trimerous flowers, corollas with valvate reduplicate aestivation, and a woody endocarp with up to 20 locules. The lack of resolution within this clade reflects a high level of similarity in the ITS sequences of the species sampled. In another study of ca. 20 species from New Caledonia and surrounding areas (Achille & Motley, unpublished), we found that the sequences are identical or differed by only a few bases, suggesting that this clade may comprise a closely related group within which rapid morphological diversification has taken place, perhaps in a manner analogous to that observed within Guettardeae as a whole. The pattern of diversification apparent among the New Caledonian species differs substantially from that of the mostly Neotropical *Guettarda* s. str. clade, where resolution is better and branches are longer (Fig. 1), even though there is comparatively little morphological variability among its members. In order to evaluate a hypothesized radiation centered on New Caledonia, it would first be necessary to sample additional species of *Antirhea* subg. *Guettardella* and subg. *Mesocarpa* (sensu Chaw & Darwin, 1992), the apparent closest relatives of New Caledonia *Guettarda*, according to our results. These subgenera are most diversified in Southeast Asia, but we were able to include only one species from South China (*Antirhea chinensis*), one from Fiji (*A. smithii*), and one from New Caledonia (*A. rhamnoides*).

The fourth group of *Guettarda* identified in our study comprises a single species, *G. acreana*, which appears as sister to *Stenostomum lucidum*. This well known *Guettarda*, widely distributed in northern South America, was sampled in our analysis because of its distinctive morphology, particularly its calyx, which is mostly caducous but not shed by abscission. Its affinities are discussed below under *Stenostomum*.

Our finding of polyphyly in *Guettarda* is consistent with morphological data, as several unique characters are associated with each of the clades revealed in our analysis (Fig. 3). *Guettarda* s. str. can be recognized by its caducous calyx, which appears to be a synapomorphy for the lineage. However, there has been some confusion between what we regard as a truly caducous calyx (shed by abscission) and a calyx that is minute or lost as it disintegrates (without leaving an evident abscission scar), as seen in *G. crispiflora*, which has been misinterpreted by several authors (e.g., Dwyer, 1980; Standley, 1931, 1934; Standley & Williams, 1975; Steyermark, 1974). Species of *Chomelia* have also been reported to possess both a deciduous and persistent calyx (Burger & Taylor, 1993), but additional developmental studies will be required to evaluate whether the nature of the calyx in this genus is homologous with that seen in *Guettarda* s. str. Our

molecular study does not provide any evidence of a close relationship between the type species of *Chomelia* and *Guettarda*, as currently circumscribed, even though, according to Taylor et al. (2004), the morphological distinction between them remains unsatisfactory. With our more thorough sampling, it also appears that the association of *Chomelia* with *G. crispiflora* suggested by Rova et al. (2002) may not reflect a close relationship between the two genera in their stricter sense and thus does not support the inclusion of *Chomelia* within *Guettarda* s. str. (as defined here).

Further analyses will be necessary before extensive nomenclatural changes can be made within *Guettarda* s.l., as most of the larger clades identified in our study are insufficiently supported in the MP bootstrap and BI analyses, and broader sampling is needed for some of them. It would, however, seem appropriate to exclude the New Caledonia species from *Guettarda*, which clearly constitute a group that is distinct from all other members of the genus, as currently circumscribed, and in particular from the type species, *G. speciosa*.

OTHER GENERA OF CORE GUETTARDEAE

Relationships within core Guettardeae, as within *Guettarda*, appear well correlated with geography. All the Paleotropical species sampled, except the Hawaiian taxa and the widespread coastal species *G. speciosa*, belong to a Paleotropical Dioecious Clade that is well supported in the BI analysis ($P = 0.99$; Fig. 2). Since the closest allies of the genera comprising core Guettardeae are Neotropical (Bremer, 1996; Bremer & Thulin, 1998; Rova et al., 2002), this suggests a New World origin for the group, with presumably two separate events involving dispersal (or vicariance) to account for the presence in the Old World of the Paleotropical Dioecious Clade and of *G. speciosa*. Dioecy characterizes all members of the Paleotropical Dioecious Clade, viz. *Timonius*, *Antirhea* and the New Caledonian *Guettarda* species, in contrast to other core Guettardeae, whose sexual system is almost always hermaphroditism, the only exception being the dioecious Hawaiian endemic genus *Bobea* (cited as polygamodioecious by Darwin & Chaw, 1990).

Timonius. Despite the presence of considerable morphological heterogeneity within *Timonius* (Wong, 1988), this genus is easily distinguished among Paleotropical Guettardeae by its free pyrenes. The five species included in our sample form a clade that is well supported in the BI analysis ($P = 1.00$), but only weakly so in the MP analysis (BS = 60%). The notion that *Timonius* is related to the endemic

Hawaiian genus *Bobea* is not confirmed by our results, despite the fact that they are both dioecious, a character found throughout the Paleotropical clade, and that they share free pyrenes, which are not known anywhere else in the tribe. *Timonius* is further distinctive among Rubiaceae because of its large number of ovary locules, which can exceed 500 in some species (Darwin, 1994), whereas most members of the family have bilocular ovaries. However, several other groups also show increases (albeit much more modest) in locule number, including some Neotropical *Guettarda*, *Bobea*, and most members of the Paleotropical Dioecious Clade. Thus, although characters of the gynoeceum are usually considered among the most important in infrafamilial classification of Rubiaceae, it seems very likely that an increase in the number of locules has occurred several times independently within Guettardeae, although the evolution of this character within the tribe may not be strictly homologous in the lineages in which it occurs.

Antirhea. Although numerous genera and species have been associated with or included in *Antirhea*, our data suggest that a restricted generic circumscription may be appropriate. The monotypic Neotropical genus *Pittoniotis* has sometimes been included in *Antirhea* (e.g., by Dwyer, 1980), but neither of our trees provides evidence for a close relationship with either *Antirhea* or the previously included Neotropical segregate genus *Stenostomum* (Fig. 2). The morphological basis traditionally invoked for this putative relationship is rather weak, essentially limited to the presence in both *Pittoniotis* and *Antirhea* of calyx-crowned drupes with few united pyrenes, characters that are in fact fairly common within core Guettardeae. *Pittoniotis* differs, however, in several important ways, such as having paniculate inflorescences, exserted stamens, and distinctive prolate 3-colporate pollen with perforate exine (the latter feature occurring also in *Stenostomum* sect. *Resinanthus* Borhidi, represented in our analysis by *S. acutatum* and *S. myrtifolium*), whereas *Antirhea* has monochasial cymes, included stamens, and oblate-spheroidal 3-colporate pollen with reticulate exine (Achille, unpublished data). All the distinctive features of *Pittoniotis* mentioned above are shared with *Malanea* (Fig. 3), but occur nowhere else within core Guettardeae (the outgroup taxa used in this study, however, exhibit some of them; Achille, pers. obs.). This suggests an affinity between *Pittoniotis* and *Malanea* that would be congruent with the sister relationship indicated by the BI analysis ($P = 0.85$).

The ITS data clearly indicate that *Antirhea* s.l. (i.e., including *Stenostomum*) is polyphyletic. Even the

more restricted definition of the genus proposed by Chaw and Darwin (1992), who included only the Paleotropical species but excluded *Stenostomum*, appears to be paraphyletic with respect to Paleotropical members of *Guettarda* and *Timonius*. Our sampling, while modest, suggests that in order to render *Antirhea* sensu Chaw and Darwin (1992) monophyletic, it would be necessary to include both *Timonius* and the New Caledonia species of *Guettarda*. If such a broad definition were used, *Antirhea* would constitute a very large genus (comprising about 230 species) that corresponds more or less to Schlechter's (1906) broad concept of *Timonius*. Adopting such a circumscription would, however, make it very difficult to define *Antirhea* based on morphological characters and thus does not seem advisable.

It would therefore seem best to limit *Antirhea* to species from the Indian Ocean, comprising *A. borbonica* (the type species) and two close relatives (*A. bifurcata* (Desf.) Hook. and *A. madagascariensis* Chaw), which together correspond to *Antirhea* sensu Jansen (1984), or *Antirhea* subg. *Antirhea* sensu Chaw and Darwin (1992). In our analysis, this group (represented by *A. borbonica* only) is sister to all the other Paleotropical Guettardeae (BS = 63%, $P = 0.99$). A more narrowly defined *Antirhea* would be easily characterized by its monomorphic male and female inflorescences (i.e., which have similar branching patterns), ovaries with only 2 or 3(4) locules, and the presence of crystals in the leaf epidermis.

If this narrower interpretation of *Antirhea* is adopted, a new generic placement would be needed for the species belonging to *Antirhea* subg. *Guettardella* (represented in our study by *A. chinensis*, the type species of *Guettardella*, and by *A. rhamnoides*) and *Antirhea* subg. *Mesocarpa* (represented by *A. smithii*). Chaw and Darwin (1992) recognized both these subgenera, which together formed a clade in their analysis. The first subgenus comprises species mostly from Malesia, Southeast Asia, and the southwest Pacific with small, 3- to 11-locular fruits, and the second includes Melanesian species with large, 8- to 16-locular fruits. Taken together, this group corresponds to *Guettardella* sensu Jansen (1984), which could be resurrected to accommodate these taxa. As the New Caledonian species of *Guettarda* may belong to the same broad clade as the *Guettardella* group (BS < 50%, $P = 0.99$), they could perhaps also be included in an even more broadly defined *Guettardella*. If this relationship is confirmed by future studies and a substantially enlarged *Guettardella* is recognized, it would, however, constitute a heterogeneous group, since the New

Caledonian species are morphologically quite variable and different from those currently included in *Guettardella*.

Stenostomum. Our results suggest that *Stenostomum* may also be polyphyletic, with *S. lucidum* in a weakly supported sister relationship to *Guettarda acreana* (BS = 52%, $P = 0.89$) and two other species (*S. acutatum* and *S. myrtifolium*) forming a separate clade (BS = 94%, $P = 1.00$) that is sister to *Chomelia* in the MP analysis (BS = 52%). As mentioned above, Borhidi and Fernandez (1993–1994) placed *S. lucidum* in *Stenostomum* sect. *Stenostomum*, whereas the other two species in our sample belong to their section *Resinanthus*. Our study supports the idea that these two groups are distinct, as suggested by morphology (Borhidi & Fernandez, 1993–1994), but their placement in different parts of our trees would not be consistent with maintaining them as a single genus. The possible sister relationship of *G. acreana* to the type species of *Stenostomum*, although weakly supported in our trees, would on the other hand be consistent with morphology, as several characters shared between these taxa (e.g., similar inflorescence structure, small ovoid fruits, and a calyx that is not shed by abscission) are not found in members of *Guettarda* s. str. For example, Bremekamp (1959), who was familiar with *Stenostomum* (under the name *Antirhea*) in Central America and the West Indies, while studying an undetermined specimen of *G. acreana* collected in Surinam, recognized it as an *Antirhea*. He consequently did not suspect any relation of his specimen to *Guettarda* and described it as a new species, *A. surinamensis* Bremek. It might thus prove appropriate to include the South American *G. acreana* within *Stenostomum*, a relationship that earlier authors (e.g., Steyermark, 1972) perhaps overlooked simply because *Stenostomum* occurs farther to the north, from the West Indies to northern Central America (Borhidi & Fernandez, 1993–1994).

Despite being well distinguished by having stipules united into a sheath, *Neolaugeria* was merged into *Stenostomum* (as a third section) by Borhidi and Fernandez (1993–1994). We are unable to confirm this interpretation, as the two species of *Neolaugeria* in our sample (which comprise a well supported pair, BS = 100%, $P = 1.00$) form a paraphyletic group with the *S. lucidum* clade in the MP tree, whereas they appear as sister to the rest of the ingroup in the BI tree, although both topologies are unsupported (BS < 50%, $P = 0.63$). Moynihan and Watson (2001) also rejected the inclusion of *Neolaugeria* in *Stenostomum* using ITS sequence data. Their results were, however, based on an analysis that included only one species of *Stenostomum*, *S. myrtifolium*, a member of section

Resinanthus, which in our study was resolved in a clade distant from the *Neolaugeria* group.

Stenostomum has been considered a synonym of *Antirhea* by some authors (e.g., Schumann, 1891; Standley, 1934; Bremekamp, 1966; Airy Shaw, 1973; Steyermark, 1974; Robbrecht, 1988) because these two genera are morphologically similar in several aspects (e.g., monochasial cymes, a stony endocarp often with few locules, and a persistent calyx). Our results suggest that the type species of *Stenostomum* is indeed closely related to *Antirhea*, and in particular to *A. borbonica* (the type of that genus), although placed sister to the Paleotropical clade. Our data are thus consistent with Chaw and Darwin's (1992) suggestion that *Stenostomum* is best treated as a distinct genus.

Bobea. The exclusion of the Hawaiian genus *Bobea* from Guettardeae was proposed by Rova (1999), but both our analyses suggest that *Bobea* is nested well within the tribe (BS = 99% and $P = 1.00$). Furthermore, the placement of *Bobea* within core Guettardeae has long been recognized based on morphological features. *Bobea* has numerous characters in common with other genera of core Guettardeae, including typically secund, monochasial inflorescences that are once-dichotomous at the base; fleshy fruits with numerous similarly-structured pyrenes; elongate cylindrical seeds with small cotyledons and topped by an obturator; and overall vegetative and floral morphology which closely resembles some species of *Antirhea*, *Guettarda*, and *Timonius*. The position of *Bobea* in the tree presented by Rova (1999) thus seems at odds with both ITS data and morphology, suggesting that Rova's placement may have resulted from an error in sampling or analysis.

Within core Guettardeae, *Bobea* does not appear to be closely related to *Timonius*, despite the fact that these two genera were once considered as synonyms (Candolle, 1830) or, more recently, as close allies (Darwin & Chaw, 1990). On the contrary, as indicated earlier, *Bobea* may instead be sister to *G. crispiflora* + *G. hirsuta*, thus forming the *Bobea* Clade, although this relationship is weakly supported in the BI analysis ($P = 0.92$) and unsupported in the MP tree (Fig. 2). It is worth noting, however, that all of these species are well-characterized (Fig. 3) by the presence of "Bobea type" pollen (spheroidal porate pollen with very thick, reticulate exine; Achille, unpublished data), which appears to be unique in the tribe, and also by their inflorescence structure (bifid monochasial cymes), distinctive from *Guettarda* s. str. A New World origin of *Bobea* may thus be plausible, since *G. crispiflora* and its relatives are distributed in the Neotropics and in particular on the Pacific coast of

Central and South America and the Cocos Islands, rather than a western Pacific origin, as implied by a traditionally assumed relationship to the Paleotropical *Timonius*. If *Bobea* is indeed derived from a Neotropical ancestor, its dioecy may have evolved independently from the Paleotropical Dioecious Clade, perhaps as an adaptation to an insular environment, a pattern that is seen in many other Hawaiian groups (Sakai et al., 1995). Hawaii has one of the highest incidences of dioecy (14.7%) of any flora in the world, and many dioecious lineages are derived from dimorphic ancestors, which was thought to be the case for *Bobea* (Sakai et al., 1995). *Bobea* may thus be a 13th Hawaiian lineage that is believed to have evolved dioecy from hermaphroditic colonists (Sakai et al., 1995).

CONCLUSIONS

Our study, using a broad sampling that represents most of the morphological and geographical diversity within *Guettarda*, indicates that the genus is polyphyletic. *Guettarda* should probably be restricted to a group of species characterized by dichasial cymes (sometimes reduced to solitary flowers) and a caducous calyx with a circular abscission zone, which is well-diversified in the Neotropics, but also comprises a single widespread species in the Paleotropics. The numerous endemic New Caledonian species currently assigned to *Guettarda* clearly belong to a distinct *Antirhea*-*Guettardella* complex, whose internal relationships are in need of further study. Our results also indicate that several morphologically distinctive Neotropical species traditionally included in *Guettarda* are placed outside *Guettarda* s. str., although their exact affinities remain unclear.

Our study also provides some interesting insights concerning the delimitations of other genera in core Guettardeae and the distribution of characters within the tribe. Our data confirm that the Neotropical genus *Stenostomum* is distinct from *Antirhea*, which should thus be considered as strictly limited to the Old World. Furthermore, *Antirhea* and *Stenostomum* both appear to be polyphyletic and may thus require the application of narrowed generic circumscriptions. Features of the gynoecium (such as the number of locules and fusion of the pyrenes), which have often been considered among the most important characters for recognizing suprageneric taxa within Rubiaceae, do not appear to be particularly well correlated with the groups identified in our analysis. Several interesting correlations have, however, been found between our phylogeny based on ITS data and the distribution of certain previously-ignored or underutilized characters. For example, a large Paleotropical

Dioecious Clade is characterized by its distinctive sexual system. The recognition of several pollen types among the members of core Guettardeae, and the homogeneity in pollen type seen within several clades identified in our phylogeny, also suggest that palynological characters may be valuable for further studies at the generic and/or specific levels within the tribe. Finally, with regard to biogeography, although *Guettarda* s.l. and *Antirhea* s.l. have generally been considered to show trans-Pacific distributions with two disjunct areas of diversification, one in the Old World and another in the Neotropics, our study suggests that this interpretation was erroneously based on non-monophyletic generic concepts.

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